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(54) Title: DELIVERY OF TREFOIL PEPTIDES

(57) Abstract: The present invention relates to a micro-organism, preferably a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, preferably a gram-positive bacterial strain, delivering a trefoil peptide*in vivo*. Preferably said trefoil peptide is TFF1. The present invention further relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of such a bacterial strain. The present invention also relates to a pharmaceutical composition comprising a trefoil peptide delivering bacterium as well as methods of treatment of acute gastro-intestinal inflammatory diseases comprising administration of said transformed bacterial strains, particularly for treating acute colitis, including but not limited to acute flare-ups of Crohn's disease and ulcerative colitis in humans, as well as for treating gastro-intestinal disorders of a similar nature in other animal species.

DELIVERY OF TREFOIL PEPTIDES

The present invention relates to the field of *in vivo* protein delivery systems. More particularly, the present invention relates to the secretion *in vivo* of trefoil peptides by micro-organisms, preferably bacterial strains, preferably non-pathogenic strains, preferably non-invasive strains, preferably food grade strains, methods for delivering trefoil peptides using said systems and the use of said trefoil peptide expression systems for treatment of inflammatory disorders of the gastro-intestinal tract.

Lactococcus lactis is a Gram-positive non-pathogenic lactic acid bacterium which can survive in the intestine (Klijn *et al.*, 1995). It is not certain whether *L. lactis* can also be metabolically active in all of these environments.

The expression of tetanus toxin fragment C by *Lactococcus lactis* in view of vaccination was described by Wells *et al.* (1993b) and Robinson *et al.* (1997). Further, it was demonstrated that when preparations of *L. lactis* bacteria engineered to express either Interleukin-2 or Interleukin-6 together with tetanus toxin fragment C (TTFC) were administered intranasally to mice, more than 10 times more anti-TTFC was produced than after similar administration of strains expressing TTFC alone (International patent application published under WO 97/14806). These results prove the use of a cytokine-secreting, non-invasive experimental bacterial vaccine vector to enhance immune responses to a co-expressed antigen. Also an approach has been described to attach heterologous protein fragments in the cell wall and by this way display them at the *L. lactis* surface, possibly leading to more enhanced vaccination properties (WO 97 09437 Steidler, Remaut, Wells).

Trefoil peptides are secreted by epithelial mucus cells and are stable in an acid environment. These peptides contribute to the protection of the mucosa (formation of a gel over the epithelium) and are probably involved in the repair of damaged mucosa by stimulation of epithelial migration (Playford *et al.*, 1996). The production of trefoil peptides increases locally in regions where damage occurs such as gastric ulcers and colitis (Wright *et al.*, 1990). Babyatsky *et al.* (1996) have shown that the administration of recombinant trefoil peptides reduces the damage at those places. In contradiction with most other proteins that are important for the protection of the mucosa (such as epidermal growth factor), most studies have demonstrated that trefoil peptides cause little or no proliferation (Playford *et al.*, 1996). Three members of this family of trefoil peptides have been identified in humans and originally designated: pS2 (breast cancer oestrogen inducible gene, O. Lefebvre, 1993), SP (spasmolytic peptide) and ITF

(intestinal trefoil factor). In the present nomenclature pS2 is renamed as TFF1, SP as TFF2 and ITF as TFF3 (see e.g. Wong *et al.*, 1999). This new nomenclature will be used throughout the present text.

In humans, mice and rat TFF1 and TFF2 are predominantly found in the stomach while TFF3 is predominantly found in the duodenum and colon. Wong *et al.* (1999) give a recent overview of trefoil peptides. The contents of this article are incorporated by reference in the present disclosure.

TFF1 is thought to act through a cell surface receptor (Tan *et al.*, 1997).

The use of trefoil proteins or peptides for treatment of disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal are described in WO 97/38712 and WO 92/14837. These proteins can be used either to treat lesions in these areas or to inhibit the formation of lesions. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, Crohn's disease, ulcerative colitis and acute colitis of chemical, bacterial or obscure origin.

Trefoil peptides are particularly useful to treat acute colitis.

ITF has also been used in combination with EGF (epidermal growth factor) for treating gastro-intestinal tract ulcers. *In vitro* and *in vivo* experiments have shown that the wound healing activities of EGF are markedly increased by treatment of EGF in combination with ITF, without increasing the proliferative action of EGF (Chinery and Playford, 1995).

Inflammatory bowel disease is the group name for a range of gastro-intestinal inflammations. Belonging to this group are enteritis, colitis, inflammations of respectively the mucosa of the duodenum or the colon. Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa) are closely related, chronic and spontaneously recurring diseases of the gastro-intestinal tract. These diseases are immunologically mediated and have environmental and genetic causes. Sartor (1995) describes the different aspects of inflammatory bowel disease. Crohn's disease has been particularly studied by for instance Herfath and Sartor, (1994), Cominelli *et al.* (1994), and MacDermott (1989).

The aim of the present invention is to provide a method for delivering trefoil peptides to treat gastro-intestinal disorders.

Another aim of the present invention is to provide a pharmaceutical composition for treating gastro-intestinal disorders.

The present invention relates more particularly to a micro-organism delivering a trefoil peptide *in vivo*. Preferentially said micro-organism is a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, more preferably a gram-positive bacterial strain, most preferably a lactic acid fermenting bacterial strain, preferably a *Lactococcus* or a *Lactobacillus* species expressing a trefoil peptide *in vivo*. The present invention is thus applicable to any of the *Lactococcus* or *Lactobacillus* species or subspecies selected from the list comprising *Lactococcus garvieae, Lactococcus lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *hordniae, Lactococcus lactis, Lactococcus lactis* subsp. *Lactis, Lactococcus piscium, Lactococcus plantarum, Lactococcus raffinolactis, Lactobacillus acetotolerans, Lactobacillus acidophilus, Lactobacillus agilis, Lactobacillus algidus, Lactobacillus alimentarius, Lactobacillus amylolyticus, Lactobacillus amylophilus, Lactobacillus amylovorus, Lactobacillus animalis, Lactobacillus aviarius, Lactobacillus aviarius* subsp. *araffinosus, Lactobacillus aviarius* subsp. *aviarius, Lactobacillus bavaricus, Lactobacillus bifermentans, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus bulgaricus, Lactobacillus carnis, Lactobacillus casei, Lactobacillus casei* subsp. *alactosus, Lactobacillus casei* subsp. *casei, Lactobacillus casei* subsp. *pseudoplantarum, Lactobacillus casei* subsp. *rhamnosus, Lactobacillus casei* subsp. *tolerans, Lactobacillus catenaformis, Lactobacillus cellobiosus, Lactobacillus collinoides, Lactobacillus confusus, Lactobacillus coryniformis, Lactobacillus coryniformis* subsp. *coryniformis, Lactobacillus coryniformis* subsp. *torquens, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus curvatus* subsp. *curvatus* , *Lactobacillus curvatus* subsp. *melibiosus, Lactobacillus delbrueckii, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus divergens, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus fornicalis, Lactobacillus fructivorans, Lactobacillus fructosus, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus graminis, Lactobacillus halotolerans, Lactobacillus hamsteri, Lactobacillus helveticus, Lactobacillus heterohiochii, Lactobacillus hilgardii, Lactobacillus homohiochii, Lactobacillus iners, Lactobacillus intestinalis, Lactobacillus jensenii, Lactobacillus johnsonii, Lactobacillus kandleri, Lactobacillus kefiri, Lactobacillus kefiranofaciens, Lactobacillus kefirgranum,*

Lactobacillus kunkeei, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus minor*, *Lactobacillus minutus*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus paracasei* subsp. *tolerans*, *Lactobacillus parakefiri*, *Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus perolens*, *Lactobacillus piscicola*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus rimae*, *Lactobacillus rogosae*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei* subsp. *carnosus*, *Lactobacillus sakei* subsp. *sakei*, *Lactobacillus salivarius*, *Lactobacillus salivarius* subsp. *salicinius*, *Lactobacillus salivarius* subsp. *salivarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus suebicus*, *Lactobacillus trichodes*, *Lactobacillus uli*, *Lactobacillus vaccinostercus*, *Lactobacillus vaginalis*, *Lactobacillus viridescens*, *Lactobacillus vitulinus*, *Lactobacillus xylosus*, *Lactobacillus yamanashiensis*, *Lactobacillus yamanashiensis* subsp. *mali*, *Lactobacillus yamanashiensis* subsp. *Yamanashiensis* and *Lactobacillus zeae*.

It was not obvious from the capacity of *Lactococcus lactis* to deliver one heterologous antigen or its ability to produce molecules such as IL-2 and IL-6 *in vitro* and *in vivo* that bacteria would be an appropriate vehicle for delivery of other types of peptides or polypeptides *in vivo*. Further it is unknown whether said trefoil peptides expressed by said bacterial strains will provide a beneficial effect to inflammatory diseases of the gastro-intestinal tract, such as inflammatory bowel disease or acute colitis.

It is, therefore, surprising that it could be demonstrated in the present Examples section that bacterial strains are able to express trefoil peptides *in vivo* when present in the gastro-intestinal canal and exert a healing effect in acute colitis situations. By way of example, PCR fragments containing the coding region mouse TFF1 were cloned. Recombinant vectors comprising these PCR clones under the control of a promotor and the *usp45 Lactococcus lactis* secretion signal sequence were constructed. Transformed *Lactococcus lactis* strains were constructed which express mouse TFF1 trefoil peptides. It was further shown in an *in vivo* mice model system that recombinant mTFF1 produced by these bacteria can surprisingly exert healing effects on the distal part of the inflamed colon.

According to a preferred embodiment, the present invention relates particularly to a bacterial strain delivering trefoil peptide *in vivo*.

According to another preferred embodiment, the present invention relates to a bacterium delivering TFF1 *in vivo*.

It is to be understood that the present invention also relates to parts or variants of any trefoil peptide. Said parts refer to biologically active parts which can be generated by methods known to those skilled in the art. These parts will generally contain at least 10 contiguous amino acids, typically at least 20 contiguous amino acids, more typically at least 30 contiguous amino acids, usually at least 40 contiguous amino acids, and preferably at least 50 contiguous amino acids. Said variants refer to variants which have the same biological activity as the above mentioned trefoil peptides.

It should also be clear that bacterial strains according to the present invention as defined above, may also express additional recombinant proteins which are beneficial to the treatment of any envisaged disorder.

According to yet another embodiment, the present invention relates to a pharmaceutical composition comprising a micro-organism expressing a trefoil peptide as defined above.

Advantageously, the pharmaceutical composition according to the present invention is preferably suitable for application to mucosal surfaces.

Pharmaceutical compositions according to the present invention, and for use in accordance to the present invention, may comprise, in addition to the micro-organism, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration. Those of relevant skill in the art are well able to prepare suitable solutions.

According to another embodiment, the present invention relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism as defined above.

According to another aspect, the present invention also relates to the use of a micro-organism as defined above for the manufacture of an agent for the delivery of trefoil peptide to the gastro-intestinal tract.

According to another embodiment, the present invention relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a micro-organism as defined above.

The present invention also relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a micro-organism delivering a TFF1 trefoil peptide *in vivo*.

The trefoil proteins expressed by the bacterial strains according to the present invention can be used either to treat lesions in these areas or to inhibit the formation of lesions caused by gastro-intestinal diseases and disorders.

The expression " gastric and/or intestinal diseases and/or disorders" relates to all types of gastric, intestinal and gastro-intestinal diseases and/or disorders. In preferred embodiments of the invention this expression relates to acute gastro-intestinal inflammatory diseases and disorders. These diseases are preferably acute gastro-intestinal disorders of chemical, bacterial or obscure origin. Belonging to this group are enteritis, colitis, including but not limited to acute flare-ups in Crohn's disease and ulcerative colitis inflammations of, respectively, the mucosa of the duodenum or the colon. Also included herewith is traveller's disease. In other prefered embodiments of the invention the expression "gastric and/or intestinal diseases and/or disorders" relates to chronic and spontaneously recurring diseases of the gastro-intestinal tract such as Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

The expression "gastric and/or intestinal diseases and/or disorders" also relates to diseases involving lesions at mucosal surfaces. As such, the disease states to be treated by the methods and pharmaceutical compositions of the invention can also include disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, and Crohn's disease.

The present invention thus relates to the use of a micro-organism as described above for the preparation of a medicament for treatment of gastric and/or intestinal diseases and/or disorders.

The present invention also relates to the use of a micro-organism as described above for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases, acute colitis, acute flare-ups of Crohn's diseases and ulcerative

colitis, and for treatment of chronic and spontaneously recurring diseases of the gastro-intestinal tract comprising Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

According to another embodiment, the invention relates to the use of a micro-organism as described above for the preparation of a medicament for inhibiting the formation of lesions caused by gastric and/or intestinal diseases and disorders.

Administration of the micro-organism may be orally or by means of any other method known in the art allowing the micro-organism to enter the desired places to be treated, such as e.g. anal, vaginal. The micro-organism may be applied in a nutrient medium, i.e. a medium containing a substance or substances which sustain (at least *in vitro*) metabolic activity of the micro-organism. Such substances may sustain viability if not growth of the micro-organism. Such substances may include an energy source such as glucose, amino acids and so on.

The individual to which the micro-organism is administrated may be a human or an animal.

In a therapeutic context, i.e. where the biological effect of delivery of the polypeptide to an individual is beneficial to that individual, administration is preferably in a 'therapeutically effective amount', this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of one symptom. The actual amount administered, and rate and time-course of administration, will depend on the aim of the administration, e.g. the biological effect sought in view of the nature and severity of the challenge and is the subject of routine optimisation. Prescriptions of treatment, for example decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition comprising micro-organisms according to the present invention may be administered in accordance with the present invention alone or in combination with other treatments, either simultaneously or sequentially.

According to another embodiment, the present invention relates to a method for producing a micro-organism delivering a trefoil peptide *in vivo* as defined above comprising transforming a micro-organism with a recombinant vector carrying a trefoil polypeptide coding sequence under the control of a suitable promoter and a suitable bacterial secretion signal sequence.

Said bacterial secretion signal sequence can be any sequence known in the art to perform said function. Preferably, for *L. lactis* said secretion signal is the *usp45 L. lactis* secretion signal sequence. Said promoter sequence can be any promoter allowing expression of said coding sequence in said micro-organism. Examples given

in the examples section include the known inducible *E. coli* phage T7 promoter and the known constitutive P1 promoter of *L. lactis*.

The present invention also relates to a recombinant vector comprising at least a part of a trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence. Said recombinant vector can be used to deliver *in vivo* at least a part of a trefoil peptide sequence which can exert on healing effect on damaged areas of the mucosal surfaces.

The present invention further relates to a recombinant vector as defined above, having a nucleotide sequence as represented by any of SEQ ID NOs 1, 2 or 4.

The following examples merely serve to illustrate the present invention, and are not to be construed as limiting the invention in any way.

All documents mentioned in this text are incorporated by reference.

FIGURE LEGENDS

Figure 1: Overview of the plasmids used.

Figure 1a : Schematic maps of the plasmids pL2mTFF1v1, and pT1mTFF1. T7 is the major late promoter from coliphage T7 (Studier and Moffatt, 1986). P1 is the lactococcal promoter as in Waterfield *et al.*, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococal Usp45 protein (van Asseldonck *et al.*, 1990), mtff1 is a DNA fragment encoding the mature part of murine TFF1, mtff1v1 is a DNA fragment encoding a truncated (missing two aminoterminal aa residues) mature murine TFF1, Cm is the chloramphenicol selection marker, Em is the erythromycin selection marker. For pPICmTFF1 : PPMF is the prepro *Saccharomyces cerevisiae* α-mating factor; AOX1 prom is the alcohol oxidase promotor; AOX1 term is the alcohol oxidase terminator; HIS4 is the Histidol dehydrogenase gene; Ori is an *Escherichia coli* origin of replication; AOXfr is a 3' fragment of the alcohol oxidase gene; AmpR is the ampicilin resistance gene, All components are from the pPIC9 plasmid (Invitrogen).

Figure 1b : DNA sequence of plasmid pL2mTFF1v1 (SEQ ID NO 1).

Figure 1c : DNA sequence of plasmid pT1mTFF1 (SEQ ID NO 2).

Figure 1d : DNA sequence of plasmid pPICmTFF1 (SEQ ID NO 3)

Figure 2: SDS-PAGE. The different protein fractions are derived from the medium of *L. lactis* MG1820 [pILPOL] (control), MG1820 [pILPOL; pL2mTFF1v1] , MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells. The two left lanes contain marker proteins wherein the molecular weight is given in kDa. The proteins were visualised using Coomassie Blue staining.

Figure 3: Representation of the histological scores of the distal part of the colon. Top left hand side graphic: epithelium damage (distal part colon). Top right hand side graphic: inflammatory infiltration (distal part colon). Bottom graphic: sum of the histological scores of the top graphics (distal part colon).

Figure 4 : Representation of the histological scores of the distal part of the colon of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment wit MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.

Figure 5 : Pro-inflammatory cytokine titrations in acute inflamed colon tissue. Interleukin-1β in distal colon (left) and interferon-γ in middle and distal colon (right) of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment wit MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.

Figure 6 : SDS-PAGE of protein fractions from the medium of selected *Pichia pastoris* (GST115::pPICmTFF1) and negative control. The mTFF1 producer clone which was further used for production of mTFF1 is indicated by an arrowhead The proteins were visualised using Coomassie Blue staining.

Figure 7 : A: Gelfiltration pattern of purified mTFF1 (Superdex 75; Pharmacia). The mTFF1 protein eluted in two peaks with the majority being present in fractions 14, 15, 16 (dimer) and 20 (monomer). The identity of the protein in these fractions was shown to be mTFF1 by SDS-PAGE (insert). The proteins were visualised using Coomassie Blue staining. B: reducing and non reducing SDS-PAGE of purified mTFF1. Left lanes are size markers of indicated sizes, coomassie brilliant blue staining.

Figure 8: Representation of the histological scores of the distal part of the colon of mice treated by intraperitoneal injection (i.p.), oral (oral) and rectal (rectal) inoculation, before (pre), during (du) or after (po) installation of acute DSS-induced colitis. DSSdu represents scores of PBS treated mice induced for acute DSS colitis.

EXAMPLES

Example 1: Cloning and expression of mouse TTF1 (mTTF1)

Culture media

GM17 is M17 (Difco, Detroit) supplemented with 0.5 w/v % of glucose. M9 medium contains per litre: 6g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 0.1 mmol of $CaCl_2$ and 5 g of Casitone (Difco). M9B is M9 supplemented with 2.1 g of $NaHCO_3$ and 2.65 g of Na_2CO_3 per liter. GM9B is M9B supplemented with 0.5 w/v % of glucose. LM9B is M9B supplemented with 0.5 w/v % of lactose.

When appropriate the antibiotics, erythromycin (Er) or chloramphenicol (Cm), were added to the respective media at final concentrations of 5 µg/ml each. The designation used to indicate the presence of antibiotic is, e.g. GM17Er, LM9BCm and so on. Solid media contained 1.2 % agar.

Recombinant DNA techniques

DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out according to standard procedures. *L. lactis* was transformed by electroporation of cells grown in the presence of glycin (Wells *et al.*, 1993a). Plasmid DNA was routinely purified using the Qiagen Plasmid Kit

PCR amplification of mTFF1

The PCR reaction was carried out on a plasmid containing mTFF1 cDNA (Lefebvre, 1993) using the oligonucleotide primers mTFF1S and mTFF1A. The mTFF1S primer corresponds to the first 18 nucleotides of the sense strand of *mTFF1* from the first nucleotide behind the signal sequence. The mTFF1A primer is complementary to the last 26 nucleotides of the sense strand of *mTFF1* including the stop codon, and introduces an extra *Spe*I restriction site.

mTFF1S: 5'-CAGGCCCAGCCCAGGCC –3' (SEQ ID NO 4)

mTFF1A: 5'-GCACTAGTTAGAAGGGACATTCTTCTTCTTG AG-3' (SEQ ID NO 5) wherein ACTAGT in mTFF1A represents an *Spe*I site

PCR amplification was carried out using Vent™ DNA polymerase (New England Biolabs (Beverly, USA) which gives a PCR product carrying blunt ends. The PCR mixture consisted of 2 units Vent DNA polymerase, 10µl Vent buffer (thermopol), 4µl dXTP's (0.5mM maximum), 5µl (0.5µM) of each primer, 1µl (50 ng) template DNA and 74µl H_2O. Six reactions were set up differing in their final concentration of $MgSO_4$, adjusted to 0, 1, 2, 3, 4 and 5 mM respectively. PCR amplification cycles were: T_0 for 300" at 94°C, T_1 for 45" at 94°C, T_2 for 30" at 60°C, T_3 for 20" at 72°C, T_4 for 10" at 20°C. These cycles T_1 until T_3 were carried out 30 times.

PCR amplification with these primers rendered the gene for mature *mTFF1* lacking the signal sequence and including an additional *Spe*I restriction site. After checking by gel electrophoresis, the amplified fragment appeared as a band in the expected length range. The 5' end of the mTFF1 sequence contains two possible target sequences complementary to the forward primer. As a consequence two fragments of 202 base pairs and 208 base pairs respectively can be amplified from the mTFF1 cDNA by use of the mentioned primers. These fragments are not expected to be resolved by agarose gel electrophoresis.

Construction of plasmids

Two different types of vectors were used as acceptors for the mTFF1 trefoil peptide encoding PCR fragment. The primary structure of the two parental vectors - pT1NX, derived from pTREX1 (Wells and Schofield, 1996), and pLET2NX, derived from pLET2N (Steidler *et al.*, 1995) - contains the following common elements: a promoter (T7 or P1), the *L.lactis usp45* secretion signal sequence (van Asseldonk *et al.*, 1990 and European patent application published under No. 0 455 280), modified to contain a *Nae*I restriction site overlapping the sequence encoding the ultimate aa residue (Steidler *et al.*, 1995), and a downstream *Spe*I restriction site. pT1NX derived plasmids specify resistance to erythromycin; pLET2NX derived plasmids specify resistance to chloramphenicol. The PCR fragments were treated for 1 hour at 37°C using 50µl DNA solution, 10µl *Spe*I-buffer, 50 units *Spe*I, 10 units T4 polynucleotide kinase (Gibco BRL, Bethesda, USA), 0.5 mM ATP, adjusted to pH 7.5, and 36µl H_2O. The vector pT1NX was digested for 1 hour at 37°C using 10 à 20µl purified DNA, 10µl *Nae*I buffer, 10 units *Nae*I, 50 units *Spe*I, 1 unit calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany) and 73 à 63µl H_2O. After 30 minutes incubation, 50 units of *Spe*I and 10 units of *Nae*I were again added to the mixture. The restriction enzymes were inactivated and extracted from the mixture by phenol/chloroform extraction. After restriction digestion, the mTFF1-derived band (comprising a 195 bp

and a 201 bp fragment as described before under "PCR amplification of mouse TFF1 (mTFF1)", and the vector parts were excised from the agarose gel. Following ligation of the respective PCR fragments and the vector for 45 minutes at 16°C using "Ready To Go" T4 DNA ligase (Pharmacia Biotech, UK) recombinant plasmids were obtained containing the mTFF1 cistron as an in-frame fusion to the *usp45* secretion signal sequence under the control of the promoter.

The plasmid pT1mTFF1 (Figure 1a), which contains the constitutive *L. lactis* P1 promoter, resulted from ligation of the purified *Nae*I - *Spe*I vector part of pT1NX and the *Spe*I cut and 5' phosphorylated PCR fragment.

The plasmid pL2mTFF1v1 (Figure1a), which contains the inducible *E. coli* phage T7 promoter, resulted from ligation of the purified *Nae*I - *Spe*I vector part of pLET2N and the *Spe*I cut and 5' phosphorylated PCR fragment. The T7 promoter can only be activated by the cognate T7 RNA polymerase encoded by e.g. plasmid pILPOL. This plasmid is present in *L. lactis* strain MG1820 [pILPOL] (Wells *et al.*, 1993c).

For structural analysis plasmid pT1mTFF1 was transformed into *L. lactis* strain MG1363. The cells were grown on GM17Er plates. Colonies were grown in 2.5 ml GM17Er and the plasmid was isolated. By means of an analytical digest, the restriction pattern of the pT1NX vector (2µl DNA (pT1NX), 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer and 15µl H_2O) and the isolated recombinant plasmid (5µl DNA, 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H_2O) were compared. The plasmids were cut with *Eco*RI and *Spe*I for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4999bp are predicted. In pT1mTFF1, two bands of 499 bp and 4999 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From each recombinant plasmid, one positive culture was streaked out on GM17Er plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Er medium and grown to saturation. The cells were collected and the plasmids were purified. Their physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* cistron had been ligated perfectly in frame with the *usp45* secretion leader sequence. pT1mTFF1 contains a 208 bp insert which represents the complete coding sequence of mature mTFF1 (as described before under "PCR amplification of mouse TFF1 (mTFF1)").

For structural analysis plasmids pL2mTFF1v1 was transformed into strain MG1820[pILPOL]. The cells were grown on GM17Cm plates. Colonies were grown in

2.5 ml GM17Cm and the plasmids were isolated. By means of an analytical digest, the restriction pattern of the pLET2NX vector (2µl DNA (pLET2NX), 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer and 15µl H_2O) and the isolated recombinant plasmid (5µl DNA, 20 units *Eco*RI, 50 units *Spe*I, 2µl *Spe*I-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H_2O) were compared. The recombinant plasmid was cut with *Eco*RI and *Spe*I for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4650bp are predicted. In pL2mTFF1, two bands of 499 bp and 4650 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From the recombinant plasmid, one positive culture was streaked out on GM17Cm plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Cm medium and grown to saturation. The cells were collected and the plasmid was purified. Its physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* cistron had been ligated in frame with the *usp45* secretion leader sequence. The analysis further showed that pL2mTFF1v1 contains a 202 bp insert (consequently missing the first two aminoterminal aa residues of mature mTFF1 ; as described before under "PCR amplification of mouse TFF1 (mTFF1)"). The sequences of the recombinant plasmids are given in figures 1b and 1c. Their complete sequences were compiled from the published sequences of the constituting parts. In addition, relevant sections of the sequences such as PCR fragments and ligation junction points were experimentally verified.

Protein expression in transformed L. lactis

L. lactis strains were transformed with the plasmids as constructed above. For transformation of the pT1mTFF1 plasmid, *L. lactis* strain MG1363 (Gasson, 1983) was used. For transformation of the pL2mTFF1v1 plasmid, *L. lactis* strain MG1820 (pILPOL) (Maeda and Gasson, 1986) was used.

The expression of the proteins by these transformed *L. lactis* strains was detected by SDS-PAGE.

To prepare culture supernatant fractions, the cells were grown for 20 hours at 28°C in five ml GM17Er medium for the pT1mTFF1 plasmid or GM17Cm medium for the pL2mTFF1v1 plasmid. The cultures were diluted 1/100 in five ml of either GM17Er or GM17Cm medium and grown for 3 hours at 28°C. The cells were collected by centrifugation at 2800 rpm for 20 min and resuspended in five ml of the appropriate medium, i.e., GM9BEr for MG1363 cells or LM9BCm for MG1820 [pILPOL] cells . After

a further five hours of growth the cells were pelleted. The proteins present in the medium fractions were recovered by phenol extraction and ethanol precipitation.

The proteins expressed in the culture supernatant fraction of a *L. lactis* MG1820 control strain compared to *L. lactis* MG1820 strains transformed with [pILPOL; pL2mTFF1v1] and L. lactis MG1363 transformed with [pTREX1; pT1mTFF1] are shown in Figure 2. This figure shows an extra protein band of the appropriate size (indicated by the arrowhead) in MG1820 [pL2mTFF1v1] and MG1363[pT1mTFF1] when compared with the controls. As can be observed from this figure, the expression of the recombinant gene is quite low. This renders the observed *in vivo* result surprising since others use purified trefoil peptides in therapies for the repair of gastric and intestinal injury at dramatically higher levels; e.g. Tran *et al.* (1999) used daily intrarectal application of human recombinant TTF2 at levels of 2.5 mg/kg body weight for five days to obtain a reduction in the inflammatory index of experimentally installed colitis in rats (intracolonic administration of dinitrobenzene sulphonic acid in alcohol).

Example 2: In vivo testing of MG1363 [pT1mTFF1]

Preparation of cells for intragastric administration

Transformants of *L. lactis* strains, MG1363 [pTREX1], MG1363 [pT1mTFF1] were streaked on GM17Er plates and grown overnight at 28°C. In each case a single colony was subsequently grown overnight at 28°C in 15 ml GM17Er medium. To this culture, 15 ml 100% glycerol was added in order to preserve said cells at –20°C. Each day, the necessary amount of cells could be inoculated for treatment of mice. To this end the culture was diluted 1/200 into 10 ml GM17Er medium. After minimum 20 hours of growth at 30°C, the cells were collected by centrifugation for 15 min at 2800 rpm. The cells were then resuspended in 1 ml M9B without antibiotic.

In vivo tests in mice with acute colitis

The effect of the trefoil peptides expressed from these *L. lactis* bacteria was tested out in mice suffering from acute colitis. Twenty-one female Balb/c mice received 5% DSS (dextrane sodium sulphate) dissolved in their drinking water during 7 days. In this manner, acute colitis was induced (Kojouharoff *et al.*, 1997). For therapeutic purposes these mice were orally inoculated daily by means of a gastric catheter using 100µl bacterial suspension (minimum 1.10^8 cells) from day 1 until day 7 of the DSS treatment. As indicated Six mice were inoculated with MG1363 [pTREX1] cells, six mice were inoculated with MG1363 [pT1mTFF1] cells and three mice were not

inoculated (DSS control). On day 8 after the induction of colitis, the mice were sacrificed and examined immunologically and histologically.

Immunological testing of the sera showed that the treated mice did not show an immune response towards the expressed proteins. Serum was taken from the mice which were bled at day 8. This serum was analysed via Western blotting to check whether it contained antibodies against the proteins present in the medium fractions of the *L. lactis* cells. The medium fractions used were derived from the *L. lactis* strains MG1363 [pTREX1] and MG1363 [pT1mTFF1]. An equivalent of 1 ml of concentrated (phenol extraction and ethanol precipitation) medium fractions were analysed by SDS-polyacrylamide (20%) gel electrophoresis. After blotting to nitrocellulose filters, the filters were incubated for 1 hour with the serum solutions of the 4 groups of mice. The serum was diluted 500 times in 20ml nitrocellulose blocking buffer (Blotto: 100ml 10x PBS, 150ml 1M NaCl, 2ml Triton X-100, 25g fat-free milkpowder, water up to a total volume of 1 liter). As a secondary antibody, sheep anti-mouse IgG coupled to horseradish peroxidase (HRP) was used. Using the 500 times diluted serum, no signal was detected.

Histological analysis was performed on colons of the treated mice. The colons were cut in the length direction and divided in three equal portions: the distal (nearest to the anus), middle and proximal parts. These colon parts were analysed histologically after an overnight fixation in 3.7% formaldehyde (in PBS), followed by paraffin embedding, ensuring upright positioning of the tissue samples in the paraffin blocks. Of each tissue sample, three parallel 3µm thick longitudinal sections, evenly spaced over the sample, were made. These crossections were coloured with hematoxylin/eosin. Histological analysis was performed in a blind fashion, meaning that the labels on the slides were covered before scoring the sections. Slides carrying sections obtained from the several groups of mice were randomized before microscopic examination. Each slide was then assigned a histological score (ranging from 0 to 5) according to the symptomatic description as defined in Table 1.

For each mouse and for each colon part, the average score of the three sections was calculated. In the distal and middle parts of the colon, the inflammation consisting of epithelial damage and infiltration were the most pronounced. In the proximal part, almost no inflammation could be observed. The average histological score was calculated for both the distal and the middle colon part per group of animals. The final histological sum score is the sum of the two separate scores (sum score = score of epithelial damage + score of infiltration) and is a measure for the degree of

the inflammation. The histological sum scores of the distal colon part for each of the groups of mice is shown in Figure 3.

From the histological scores for the distal part of the colon as set out in Figure 3, it could be concluded that there is a clear decrease of inflammation upon inoculation of mice with *L. lactis* cells producing trefoil peptides. Mice having received [pT1mTFF1] transformed *L. lactis* cells show a significant reduction of the inflammation of more than 65%.

As can be seen from Figure 3, the inflammatory infiltration and the epithelial damage in the distal part of the colon are significantly decreased following inoculation with recombinant *L. lactis* strains which secrete mTFF1 polypeptide

These results were confirmed in a separate experiment which was conducted equally, including larger groups (group size = 10) and more control groups. Figure 4 shows histological scores (obtained as described above) of healthy control mice (control) and of mice which received DSS as described, either left untreated (DSS) or treated (as described above) with MG1363, MG1363 [pT1TREX1] or MG1363 [pT1mTFF1] as indicated. The experiment shows a clear and significant decrease in the intestinal inflammation in the group of mice treated with MG1363 [pT1mTFF1]

The latter experiment was also evaluated by determining the levels of interleukin-1β (IL-1β) and interferon-γ (IFN-γ), both pro-inflammatory cytokines well known to the skilled. Mice (n=10) were inoculated with the strains indicated as described. Control = healthy mice, DSS = mice receiving 5% DSS in the drinking water without any treatment. The colon was prepared out and areas with equal surface were isolated by means of a punch (Ø = 4 mm). The tissue samples of each group were overlayed with 500 µl RPMI + 10% fetal calf serum and incubated overnight at 37°C. The supernatant was collected and titrated for cytokine content by ELISA. The amount of IL-1β and IFN-γ in the respective tissues is shown in Figure 5. The results show a clear reduction in these pro-inflammatory cytokines in groups of mice treated with MG1363 [pT1mTFF1]

Example 3: Comparison of treatment with MG1363 [pT1TFF1] and purified TFF1

Construction of plasmids

For the expression of mTFF1 form *Pichia pastoris* we constructed the plasmid pPICmTFF1. For this, the mTFF1 gene was PCR amplified as described (PCR amplification of mouse TFF1). This fragment was ligated in the opened *Nae*I restriction site of a derivative of pPIC9 (Invitrogen). The ligation mixture is transformed

to *E. coli* MC1061 and correctly assembled clones were identified by restriction analysys and DNA sequencing (sequence as in Figure 1d). In the resulting plasmid pPICmTFF1, the mTFF1 sequence is fused in frame with the *Sacharomuces cerevisiae* α-mating factor prepro secretion signal

Expression and Purification of mTFF1

The plasmid pPICmFF1 was transferred to *Pichia pastoris* GST115 by a method as described in Logghe (1995) and positive clones, which had the mTFF1 unit integrated in the his4 locus, were selected by PCR identification. These positive clones were induced with methanol and screened for expression by protein analysis of culture supernatant and one clone which showed, when compared to the negative control (negative), a particularly high expression of an extra band at 6,5 kDa (GST115::pPICmTFF1) was retained for further work (Figure 6, indicated by arrowhead). The extra protein band was identified as mTFF1 by protein sequencing. The expression procedure was optimised scaled up and optimised to a 16 l culture and mTFF1 was purified from the culture supernatant.

For this, methanol induced GST115::pPICmTFF1 supernatans was concentrated by tangential filtration (Millipore proflux M12, cut off 3000 Da) and was dialysed to pH 7.4 in a 0.02 M phosphate buffer. mTFF1 was purified from this concentrate on an ion-exchange column (Q-column of Biorad). The proteins were eluted form the column by an isocrational salt gradient. The resultant mTFF1 was more than 99% pure and was further concentrated. The final preparation contains less than 160 ng LPS /ml This amount of LPS is within acceptable limits and the pS2 protein can be used in future experiments.

Following analysis on a size exclusion column of purified mTFF1 (Superdex 75; Pharmacia) we conclude that 7.5 % of the mTFF1 is in the monomeric form, and 92.5 % is in the dimeric form (Figure 7A). This was confirmed by reducing versus non reducing SDS-PAGE of the purified mTFF1 (Figure 7B).

Assessment of biological activity of purified TFF1

A well know feature of TFF1 protein is that after administration of the protein to Caco-2 cell monolayers it significantly lowers the surface expression of E-cadherine (Liu *et al.*, 1997). We showed a lowering of 10 % of the E-cadherine surface expression after the above described preparation of mTFF1 was administred to Caco-2 monolayers.

Treatment of murine acute colitis with purified mTFF1:

For induction of acute colitis mice received 6% dextran sulfate sodium (DSS, MW 40 000) dissolved in drinking water for 7 days (Kojouharoff et al., 1997). Mice used for experiments were age-matched and had received DSS treatment simultaneously. For therapeutic purposes, mice were treated daily with 50 µg mTFF1 in 200 µl PBS before DSS administration from day –7 to 0 (pre-treatment groups), during DSS administration from day 0 to 7 (during-treatment groups) and after DSS administration from day 7 to 14 (post-treatment groups). To study different routes to deliver mTFF1, mice were treated by intraperitoneal (i.p.) injection, intragastric inoculation and rectal administration in each setup. Mice were killed on day 8 after receiving drinking water without DSS for one day (pre-treatment and during-treatment groups) and on day 14 after receiving drinking water without DSS for seven days (post-treatment groups). Non-treated control groups with DSS in drinking water were killed on day 8 and day 14. All groups consisted of 9 mice. Results are represented in Figure 8 and clearly show that in no treatment regime any statistically significant improvement can be observed. This renders the described invention surprising since a clear improvement has been observed (Figure 3 and 4). This means that the delivery of TFF1 through *L. lactis* makes an essential contribution to the observed therapeutic effect.

Table 1. ***Symptomatic description of histological scores.***

Score	Epithelium damage	Inflammatory infiltration*
0	Normal morphology	No infiltration
1	Loss of a few goblet cells	Infiltration around the basis of the crypts
2	Widespread loss of goblet cells	Infiltration which reaches the Lamina muscularis mucosae
3	Loss of crypts	Extensive infiltration which reaches the Lamina muscularis mucosae and thickening of the mucosa with prominent oedema
4	Widespread loss of crypts	Infiltration which reaches the Lamina submucosa

* Inflammatory infiltration includes infiltration of the granulocytes, macrophages and lymphocytes.

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CLAIMS

1. A recombinant micro-organism delivering a trefoil peptide *in vivo.*

2. A micro-organism according to claim 1, wherein said micro-organism is a bacterial strain.

3. A micro-organism according to claim 2, wherein said micro-organism is a food grade bacterial strain, preferably a gram-positive bacterial strain.

4. A micro-organism according to claim 3, wherein said bacterial strain is a *Lactococcus* or a *Lactobacillus species.*

5. A micro-organism according to claim 4, wherein said bacterial strain is *Lactococcus lactis.*

6. A micro-organism according to any of claims 1 to 5 wherein said trefoil peptide is TFF1.

7. Pharmaceutical composition comprising a micro-organism according to any of claims 1 to 6.

8. Method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism according to any of claims 1 to 6.

9. Use of a micro-organism according to any of claims 1 to 6 for the manufacture of an agent for the delivery of a trefoil peptide to the gastro-intestinal tract.

10. Method of treatment of gastric and/or intestinal diseases and/or disorders comprising the administration of a micro-organism according to any of claims 1 to 6.

11. Method of treatment of lesions caused by gastric and/or intestinal diseases and/or disorders comprising the administration of a micro-organism according to any of claims 1 to 6

12. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of gastric and/or -intestinal diseases and/or disorders

13. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases comprising acute colitis, acute flare-ups of Crohn's diseases and ulcerative colitis.

14. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of chronic and spontaneously recurring diseases of the gastro-intestinal tract comprising Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

15. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for inhibiting the formation of lesions caused by gastric and/or intestinal diseases and disorders.

16. Method for producing a micro-organism according to any of claims 1 to 6 comprising transforming a micro-organism with a recombinant vector carrying a trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence.

17. Recombinant vector comprising a trefoil peptide coding sequence under the control of a suitable promoter sequence and a suitable secretion signal sequence.

18. Recombinant vector according to claim 17, having a nucleotide sequence as represented by any of SEQ ID NOs 1, 2, or 3.

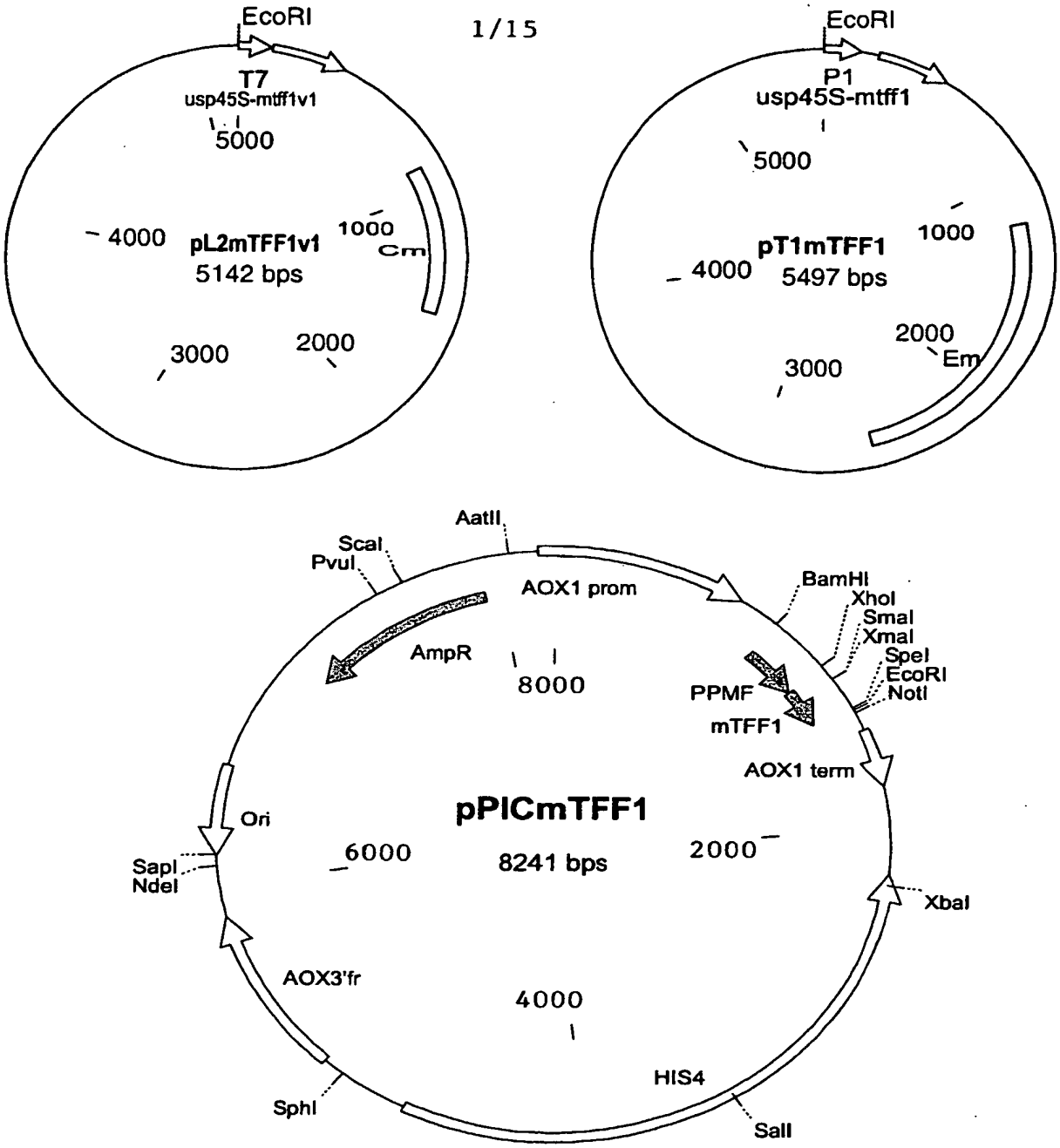
EcoRI
T7
usp45S-mtff1v1
5000
4000
pL2mTFF1v1
5142 bps
1000
Cm
3000
2000
EcoRI
P1
usp45S-mtff1
5000
pT1mTFF1
5497 bps
4000
1000
2000
Em
3000
AatII
ScaI
PvuI
AOX1 prom
BamHI
XhoI
SmaI
XmaI
SpeI
EcoRI
NotI
AmpR
8000
PPMF
mTFF1
AOX1 term
pPICmTFF1
Ori
6000
8241 bps
2000
SapI
NdeI
XbaI
AOX3'fr
4000
HIS4
SphI
SalI

Figure 1a

Figure 1b:
pL2mTFF1v1 (SEQ ID NO 1)

GAATTCGAGCTCGGTACCCGGGGATCTCGATCCCGCGAAATTAATACGACTC
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CAGGCCCAGGCCCAGGAAGAAACATGTATCATGGCCCCCCGGGAGAGGATAA
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ACTGCATAACTCATATGTAAATCGCTCCTTTTTAGGTGGCACAAATGTGAGG
CATTTTCGCTCTTTCCGGCGAGGCTAGTTACCCTTAAGTTATTGGTATGACT
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AGTCATTAGGCCTATCTGACAATTCCTGAATAGAGTTCATAAACAATCCTGC
ATGATAACCATCACAAACAGAATGATGTACCTGTAAAGATAGCGGTAAATAT
ATTGAATTACCTTTATTAATGAATTTTCCTGCTGTAATAATGGGTAGAAGGT
AATTACTATTATTATTGATATTTAAGTTAAACCCAGTAAATGAAGTCCATGG
AATAATAGAAAGAGAAAAAGCATTTTCAGGTATAGGTGTTTTGGGAAACAAT
TTCCCCGAACCATTATATTTCTCTACATCAGAAAGGTATAAATCATAAAACT
CTTTGAAGTCATTCTTTACAGGAGTCCAAATACCAGAGAATGTTTTAGATAC
ACCATCAAAAATTGTATAAAGTGGCTCTAACTTATCCCAATAACCTAACTCT
CCGTCGCTATTGTAACCAGTTCTAAAAGCTGTATTTGAGTTTATCACCCTTG
TCACTAAGAAAATAAATGCAGGGTAAAATTTATATCCTTCTTGTTTTATGTT
TCGGTATAAAACACTAATATCAATTTCTGTGGTTATACTAAAAGTCGTTTGT
TGGTTCAAATAATGATTAAATATCTCTTTTCTCTTCCAATTGTCTAAATCAA
TTTTATTAAAGTTCATTTGATATGCCTCCTAAATTTTTATCTAAAGTGAATT
TAGGAGGCTTACTTGTCTGCTTTCTTCATTAGAATCAATCCTTTTTTAAAAG
TCAATATTACTGTAACATAAATATATATTTTAAAAATATCCCACTTTATCCA
ATTTTCGTTTGTTGAACTAATGGGTGCTTTAGTTGAAGAATAAAGACCACAT
TAAAAAATGTGGTCTTTTGTGTTTTTTTAAAGGATTTGAGCGTAGCGAAAAA
TCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGGATAGACTG
TAACATTCTCACGCATAAAATCCCCTTTCATTTTCTAATGTAAATCTATTAC
CTTATTATTAATTCAATTCGCTCATAATTAATCCTTTTTCTTATTACGCAAA
ATGGCCCGATTTAAGCACACCCTTTATTCCGTTAATGCGCCATGACAGCCAT
GATAATTACTAATACTAGGAGAAGTTAATAAATACGTAACCAACATGATTAA
CAATTATTAGAGGTCATCGTTCAAAATGGTATGCGTTTTGACACATCCACTA
TATATCCGTGTCGTTCTGTCCACTCCTGAATCCCATTCCAGAAATTCTCTAG
CGATTCCAGAAGTTTCTCAGAGTCGGAAAGTTGACCAGACATTACGAACTGG
CACAGATGGTCATAACCTGAAGGAAGATCTGATTGCTTAACTGCTTCAGTTA
AGACCGAAGCGCTCGTCGTATAACAGATGCGATGATGCAGACCAATCAACAT
GGCACCTGCCATTGCTACCTGTACAGTCAAGGATGGTAGAAATGTTGTCGGT
CCTTGCACACGAATATTACGCCATTTGCCTGCATATTCAAACAGCTCTTCTA
CGATAAGGGCACAAATCGCATCGTGGAACGTTTGGGCTTCTACCGATTTAGC
AGTTTGATACACTTTCTCTAAGTATCCACCTGAATCATAAATCGGCAAAATA
GAGAAAAATTGACCATGTGTAAGCGGCCAATCTGATTCCACCTGAGATGCAT
AATCTAGTAGAATCTCTTCGCTATCAAAATTCACTTCCACCTTCCACTCACC
GGTTGTCCATTCATGGCTGAACTCTGCTTCCTCTGTTGACATGACACACATC
ATCTCAATATCCGAATAGGGCCCATCAGTCTGACGACCAAGAGAGCCATAAA
CACCAATAGCCTTAACATCATCCCCATATTTATCCAATATTCGTTCCTTAAT
TTCATGAACAATCTTCATTCTTTCTTCTCTAGTCATTATTATTGGTCCATTC

Figure 1b – continued -

ACTATTCTCATTCCCTTTTCAGATAATTTTAGATTTGCTTTTCTAAATAAGA
ATATTTGGAGAGCACCGTTCTTATTCAGCTATTAATAACTCGTCTTCCTAAG
CATCCTTCAATCCTTTTAATAACAATTATAGCATCTAATCTTCAACAAACTG
GCCCGTTTGTTGAACTACTCTTTAATAAAATAATTTTTCCGTTCCCAATTCC
ACATTGCAATAATAGAAAATCCATCTTCATCGGCTTTTTCGTCATCATCTGT
ATGAATCAAATCGCCTTCTTCTGTGTCATCAAGGTTTAATTTTTTATGTATT
TCTTTTAACAAACCACCATAGGAGATTAACCTTTTACGGTGTAAACCTTCCT
CCAAATCAGACAAACGTTTCAAATTCTTTTCTTCATCATCGGTCATAAAATC
CGTATCCTTTACAGGATATTTTGCAGTTTCGTCAATTGCCGATTGTATATCC
GATTTATATTTATTTTTCGGTATTTTTTATTAAAACGTCTCAAAATCGTTTC
TGGGACGTTTTAGCGTTTATTTCGTTTAGTTATCGGCATAATCGTTAAAACA
GGCGTTATCGTAGCGTAAAAGCCCTTGAGCGTAGCGTGCTTTGCAGCGAAGA
TGTTGTCTGTTAGATTATGAAAGCCGATGACTGAATGAAATAATAAGCGCAG
CGTCCTTCTATTTCGGTTGGAGGAGGCTCAAGGGAGTTTGAGGGAATGAAAT
TCCCTCATGGGTTTGATTTTAAAAATTGCTTGCAATTTTGCCGAGCGGTAGC
GCTGGAAAATTTTTGAAAAAAATTTGGAATTTGGAAAAAAATGGGGGGAAAG
GAAGCGAATTTTGCTTCCGTACTACGACCCCCCATTAAGTGCCGAGTGCCAA
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TTCAATCGCCAACGAATCGCCAACGTTTTCGCCAACGTTTTTTATAAATCTA
TATTTAAGTAGCTTTATTGTTGTTTTTATGATTACAAAGTGATACACTAATT
TTATAAAATTATTTGATTGGAGTTTTTTAAATGGTGATTTCAGAATCGAAAA
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GGCGAAACAAAAAGGTTTTTCAAAATCTGCGGTTGCGGCGTTAGCTATAGAA
GAATATGCAAGAAAGGAATCAGAACAAAAAAAATAAGCGAAAGCTCGCGTTT
TTAGAAGGATACGAGTTTTCGCTACTTGTTTTTGATAAGGTAATATATCATG
GCTATTAAAAATACTAAAGCTAGAAATTTTGGATTTTTATTATATCCTGACT
CAATTCCTAATGATTGGAAAGAAAAATTAGAGAGTTTGGGCGTATCTATGGC
TGTCAGTCCTTTACACGATATGGACGAAAAAAAAGATAAAGATACATGGAAT
AGTAGTGATGTTATACGAAATGGAAAGCACTATAAAAAACCACACTATCACG
TTATATATATTGCACGAAATCCTGTAACAATAGAAAGCGTTAGGAACAAGAT
TAAGCGAAAATTGGGGAATAGTTCAGTTGCTCATGTTGAGATACTTGATTAT
ATCAAAGGTTCATATGAATATTTGACTCATGAATCAAAGGACGCTATTGCTA
AGAATAAACATATATACGACAAAAAAGATATTTTGAACATTAATGATTTTGA
TATTGACCGCTATATAACACTTGATGAAAGCCAAAAAAGAGAATTGAAGAAT
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TGGCTTTTATTCGCCTTAGGGGAGCGGAGTTTGGAATTTTAAATACGAATGA
TGTAAAAGATATTGTTTCAACAAACTCTAGCGCCTTTAGATTATGGTTTGAG
GGCAATTATCAGTGTGGATATAGAGCAAGTTATGCAAAGGTTCTTGATGCTG
AAACGGGGGAAATAAAATGACAAACAAAGAAAAAGAGTTATTTGCTGAAAAT
GAGGAATTAAAAAAAGAAATTAAGGACTTAAAAGAGCGTATTGAAAGATACA
GAGAAATGGAAGTTGAATTAAGTACAACAATAGATTTATTGAGAGGAGGGAT
TATTGAATAAATAAAAGCCCCCCTGACGAAAGTCGCGACTTCGTTCTTTTTT
TACCTCTCGGTTATGAGTTAGTTCAAATTCGTTCTTTTTAGGTTCTAAATCG
TGTTTTTCTTGGAATTGTGCTGTTTTATCCTTTACCTTGTCTACAAACCCCT
TAAAAACGTTTTTAAAGGCTTTTAAGCCGTCTGTACGTTCCTTAAG

Figure 1c:
pT1mTFF1 (SEQ ID NO 2)

GAATTCGATTAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAA
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ATTTATTATCTACTCCTTTGTAGATATGTTATAATACAAGTATCAGATCTGG
GAGACCACAACGGTTTCCCACTAGAAATAATTTTGTTTAACTTTAGAAAGGA
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TACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCCCAGGCCCAGGCCCA
GGCCCAGGCCCAGGAAGAAACATGTATCATGGCCCCCCGGGAGAGGATAAAT
TGTGGCTTCCCCGGTGTCACCGCCCAGCAGTGCACGGAGAGAGGTTGCTGTT
TTGATGACAGTGTCCGGGGATTCCCGTGGTGCTTCCACCCCATGGCCATCGA
GAACACTCAAGAAGAAGAATGTCCCTTCTAACTAGTAGATCCGGCTGCTAAC
AAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAG
CATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGG
AGGAACTATATCCGGATGACCTGCAGGCAAGCTCTAGAATCGATACGATTTT
GAAGTGGCAACAGATAAAAAAAAGCAGTTTAAAATTGTTGCTGAACTTTTAA
AACAAGCAAATACAATCATTGTCGCAACAGATAGCGACAGAGAAGGCGAAAA
CATTGCCTGGTCGATCATTCATAAAGCAAATGCCTTTTCTAAAGATAAAACG
TATAAAAGACTATGGATCAATAGTTTAGAAAAAGATGTGATCCGTAGCGGTT
TTCAAAATTTGCAACCAGGAATGAATTACTATCCCTTTTATCAAGAAGCGCA
AAAGAAAAACGAAATGATACACCAATCAGTGCAAAAAAAGATATAATGGGAG
ATAAGACGGTTCGTGTTCGTGCTGACTTGCACCATATCATAAAAATCGAAAC
AGCAAAGAATGGCGGAAACGTAAAAGAAGTTATGGAAATAAGACTTAGAAGC
AAACTTAAGAGTGTGTTGATAGTGCAGTATCTTAAAATTTTGTATAATAGGA
ATTGAAGTTAAATTAGATGCTAAAAATTTGTAATTAAGAAGGAGTGATTACA
TGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACT
CAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAA
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AGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAA
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CAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGTATTCCTTACCATT
TAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACAT
CTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGA
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TGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAA
ACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACG
TACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAA
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TACTTATGAGCAAGTATTGTCTATTTTTAATAGTTATCTATTATTTAACGGG
AGGAAATAATTCTATGAGTCGCTTTTGTAAATTTGGAAAGTTACACGTTACT
AAAGGGAATGTAGATAAATTATTAGGTATACTACTGACAGCTTCCAAGGAGC
TAAAGAGGTCCCTAGCGCTCTTATCATGGGGAAGCTCGGATCATATGCAAGA
CAAAATAAACTCGCAACAGCACTTGGAGAAATGGGACGAATCGAGAAAACCC
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GGACAACGTGGAGAATTTAGAGAACGTGCTCTCCAAGACCAGTTACAAAGAG
CTAGTGCACTAAACATAATTATTAACGCTATAAGTGTGTGGAACACTGTATA
TATGGAAAAAGCCGTAGAAGAATTAAAAGCAAGAGGAGAATTTAGAGAAGAT
TTAATGCCATATGCGTGGCCGTTAGGATGGGAACATATCAATTTTCTTGGAG
AATACAAATTTGAAGGATTACATGACACTGGGCAAATGAATTTACGTCCTTT
ACGTATAAAAGAGCCGTTTTATTCTTAATATAACGGCTCTTTTTATAGAAAA
AATCCTTAGCGTGGTTTTTTTCCGAAATGCTGGCGGTACCCCAAGAATTAGA
AATGAGTAGATCAAATTATTCACGAATAGAATCAGGAAAATCAGATCCAACC
ATAAAAACACTAGAACAAATTGCAAAGTTAACTAACTCAACGCTAGTAGTGG
ATTTAATCCCAAATGAGCCAACAGAACCAGAGCCAGAAACAGAATCAGAACA
AGTAACATTGGATTTAGAAATGGAAGAAGAAAAAAGCAATGACTTCGTGTGA

Figure 1c – continued -

ATAATGCACGAAATCGTTGCTTATTTTTTTTTAAAAGCGGTATACTAGATAT
AACGAAACAACGAACTGAATAGAAACGAAAAAAGAGCCATGACACATTTATA
AAATGTTTGACGACATTTTATAAATGCATAGCCCGATAAGATTGCCAAACCA
ACGCTTATCAGTTAGTCAGATGAACTCTTCCCTCGTAAGAAGTTATTTAATT
AACTTTGTTTGAAGACGGTATATAACCGTACTATCATTATATAGGGAAATCA
GAGAGTTTTCAAGTATCTAAGCTACTGAATTTAAGAATTGTTAAGCAATCAA
TCGGAAATCGTTTGATTGCTTTTTTTGTATTCATTTATAGAAGGTGGAGTTT
GTATGAATCATGATGAATGTAAAACTTATATAAAAAATAGTTTATTGGAGAT
AAGAAAATTAGCAAATATCTATACACTAGAAACGTTTAAGAAAGAGTTAGAA
AAGAGAAATATCTACTTAGAAACAAAATCAGATAAGTATTTTTCTTCGGAGG
GGGAAGATTATATATATAAGTTAATAGAAAATAACAAAATAATTTATTCGAT
TAGTGGAAAAAAATTGACTTATAAAGGAAAAAAATCTTTTTCAAAACATGCA
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TTATAGGATTTATAGGAAAGGAGAACAGCTGAATGAATATCCCTTTTGTTGT
AGAAACTGTGCTTCATGACGGCTTGTTAAAGTACAAATTTAAAAATAGTAAA
ATTCGCTCAATCACTACCAAGCCAGGTAAAAGCAAAGGGGCTATTTTTGCGT
ATCGCTCAAAATCAAGCATGATTGGCGGTCGTGGTGTTGTTCTGACTTCCGA
GGAAGCGATTCAAGAAAATCAAGATACATTTACACATTGGACACCCAACGTT
TATCGTTATGGAACGTATGCAGACGAAAACCGTTCATACACGAAAGGACATT
CTGAAAACAATTTAAGACAAATCAATACCTTCTTTATTGATTTTGATATTCA
CACGGCAAAAGAAACTATTTCAGCAAGCGATATTTTAACAACCGCTATTGAT
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ATTTTGTTTTAGAAACGCCAGTCTATGTGACTTCAAAATCAGAATTTAAATC
TGTCAAAGCAGCCAAAATAATTTCGCAAAATATCCGAGAATATTTTGGAAAG
TCTTTGCCAGTTGATCTAACGTGTAATCATTTTGGTATTGCTCGCATACCAA
GAACGGACAATGTAGAATTTTTTGATCCTAATTACCGTTATTCTTTCAAAGA
ATGGCAAGATTGGTCTTTCAAACAAACAGATAATAAGGGCTTTACTCGTTCA
AGTCTAACGGTTTTAAGCGGTACAGAAGGCAAAAAACAAGTAGATGAACCCT
GGTTTAATCTCTTATTGCACGAAACGAAATTTTCAGGAGAAAAGGGTTTAAT
AGGGCGTAATAACGTCATGTTTACCCTCTCTTTAGCCTACTTTAGTTCAGGC
TATTCAATCGAAACGTGCGAATATAATATGTTTGAGTTTAATAATCGATTAG
ATCAACCCTTAGAAGAAAAAGAAGTAATCAAAATTGTTAGAAGTGCCTATTC
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TGGGTATCAAGTGATTTAACCAGTAAAGATTTATTTGTCCGTCAAGGGTGGT
TTAAATTCAAGAAAAAAAGAAGCGAACGTCAACGTGTTCATTTGTCAGAATG
GAAAGAAGATTTAATGGCTTATATTAGCGAAAAAAGCGATGTATACAAGCCT
TATTTAGTGACGACCAAAAAAGAGATTAGAGAAGTGCTAGGCATTCCTGAAC
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TATGATACAGGCTGAAAATAAAACCCGCACTATGCCATTACATTTATATCTA
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AAACTTTTAGTTGGCTTGGACTGAACGAAGTGAGGGAAAGGCTACTAAAACG
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ATAGATTTATTGAATAGGTCATTTAAGTTGAGCATATTAGAGGAGGAAAATC
TTGGAGAAATATTTGAAGAACCCGATTACATGGATTGGATTAGTTCTTGTGG
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CTTGTTGTTAGTATTTGCTAGTCAAAGTGATTAAATA

Figure 1d:
pPICmTFF1 (SEQ ID NO 3)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCAT
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ACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTT
GATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCTGTC
TATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCAACAAGCTCCGCATTACAC
CCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGG
CCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGA
TGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGC
CATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCG
CAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAAACACCCGCT
TTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGAT
AGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA
GGAAGCTGCCCTGTCTTAAACCTTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAA
CTAATTATTCGAAGGATCCAAACGATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCA
TCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAA
GCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGC
ACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTA
TCTCTCGAGAAAAGAGAGGCTGAAGCCCAGGCCCAGGCCCAGGCCCAGGCCCAGGAAGAAACATGT
ATCATGGCCCCCCGGGAGAGGATAAATTGTGGCTTCCCCGGTGTCACCGCCCAGCAGTGCACGGAG
AGAGGTTGCTGTTTTGATGACAGTGTCCGGGGATTCCCGTGGTGCTTCCACCCCATGGCCATCGAG
AACACTCAAGAAGAAGAATGTCCCTTCTAACTAGTGGCGTAGAATTCCCTAGGGCGGCCGCGAATT
AATTCGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTA
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ACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCTGGATGCTGTAGG
CATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCGGGATATCGTCCATTCCGACAGCATCGC
CAGTCACTATGGCGTGCTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGG
AGCACTGTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAGCCACTATCGA
CTACGCGATCATGGCGACCACACCCGTCCTGTGGATCTATCGAATCTAAATGTAAGTTAAAATCTC
TAAATAATTAAATAAGTCCCAGTTTCTCCATACGAACCTTAACAGCATTGCGGTGAGCATCTAGAC
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GTGAAGTGATGAACTTCTGGAAGGTTGCAGTGTTAACTCCGCTGTATTGACGGGCATATCCGTACG
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AGTGTGCAATACACTTGCGTACAATTTCAACCCTTGGCAACTGCACAGCTTGGTTGTGAACAGCAT
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CTAGAACTTCAGAAGGCCCAGCAGGCATGTCAATACTACACAGGGCTGATGTGTCATTTTGAACCA
TCATCTTGGCAGCAGTAACGAACTGGTTTCCTGGACCAAATATTTTGTCACACTTAGGAACAGTTT
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CCTTGTGGGCAACGTAGATGACTTCTGGGGTAAGGGTACCATCCTTCTTAGGTGGAGATGCAAAAA
CAATTTCTTTGCAACCAGCAACTTTGGCAGGAACACCCAGCATCAGGGAAGTGGAAGGCAGAATTG
CGGTTCCACCAGGAATATAGAGGCCAACTTTCTCAATAGGTCTTGCAAAACGAGAGCAGACTACAC
CAGGGCAAGTCTCAACTTGCAACGTCTCCGTTAGTTGAGCTTCATGGAATTTCCTGACGTTATCTA
TAGAGAGATCAATGGCTCTCTTAACGTTATCTGGCAATTGCATAAGTTCCTCTGGGAAAGGAGCTT
CTAACACAGGTGTCTTCAAAGCGACTCCATCAAACTTGGCAGTTAGTTCTAAAAGGGCTTTGTCAC
CATTTTGACGAACATTGTCGACAATTGGTTTGACTAATTCCATAATCTGTTCCGTTTTCTGGATAG
GACGACGAAGGGCATCTTCAATTTCTTGTGAGGAGGCCTTAGAAACGTCAATTTTGCACAATTCAA
TACGACCTTCAGAAGGGACTTCTTTAGGTTTGGATTCTTCTTTAGGTTGTTCCTTGGTGTATCCTG

Figure 1d – continued -

GCTTGGCATCTCCTTTCCTTCTAGTGACCTTTAGGGACTTCATATCCAGGTTTCTCTCCACCTCGT
CCAACGTCACACCGTACTTGGCACATCTAACTAATGCAAAATAAAATAAGTCAGCACATTCCCAGG
CTATATCTTCCTTGGATTTAGCTTCTGCAAGTTCATCAGCTTCCTCCCTAATTTTAGCGTTCAACA
AAACTTCGTCGTCAAATAACCGTTTGGTATAAGAACCTTCTGGAGCATTGCTCTTACGATCCCACA
AGGTGGCTTCCATGGCTCTAAGACCCTTTGATTGGCCAAAACAGGAAGTGCGTTCCAAGTGACAGA
AACCAACACCTGTTTGTTCAACCACAAATTTCAAGCAGTCTCCATCACAATCCAATTCGATACCCA
GCAACTTTTGAGTTGCTCCAGATGTAGCACCTTTATACCACAAACCGTGACGACGAGATTGGTAGA
CTCCAGTTTGTGTCCTTATAGCCTCCGGAATAGACTTTTTGGACGAGTACACCAGGCCCAACGAGT
AATTAGAAGAGTCAGCCACCAAAGTAGTGAATAGACCATCGGGGCGGTCAGTAGTCAAAGACGCCA
ACAAAATTTCACTGACAGGGAACTTTTTGACATCTTCAGAAAGTTCGTATTCAGTAGTCAATTGCC
GAGCATCAATAATGGGGATTATACCAGAAGCAACAGTGGAAGTCACATCTACCAACTTTGCGGTCT
CAGAAAAAGCATAAACAGTTCTACTACCGCCATTAGTGAAACTTTTCAAATCGCCCAGTGGAGAAG
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GCTGGTCAGCAGCATAGGGAAACACGGCTTTTCCTACCAAACTCAAGGAATTATCAAACTCTGCAA
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CGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAG
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AGACAGTAAATGAAGTCCCACCAATAAAGAAATCCTTGTTATCAGGAACAAACTTCTTGTTTCGAA
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CGTTGCTATTTCGTTCAAACCATTCCGAATCCAGAGAAATCAAAGTTGTTTGTCTACTATTGATCC
AAGCCAGTGCGGTCTTGAAACTGACAATAGTGTGCTCGTGTTTTGAGGTCATCTTTGTATGAATAA
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CATCAACTTGAGGGGCACTATCTTGTTTTAGAGAAATTTGCGGAGATGCGATATCGAGAAAAAGGT
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GAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTG
TCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG
GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCC
TTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC
ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCT
AACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA
AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGC
AAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCT
GACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC
ACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGG
TCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGT

Figure 1d – continued -

GCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCC
GGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGC
ATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGA
AGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATG
CCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATG
CGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTA
AAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGA
TCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTT
TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGT
TGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGC
GGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAA
GTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACG
AGGCCCTTTCGTCTTCAAGAATTAATTCTCATGTTTGACAGCTTATCATCGATAAGCTGACTCATG
TTGGTATTGTGAAATAGACGCAGATCGGGAACACTGAAAAATAACAGTTATTATTCG

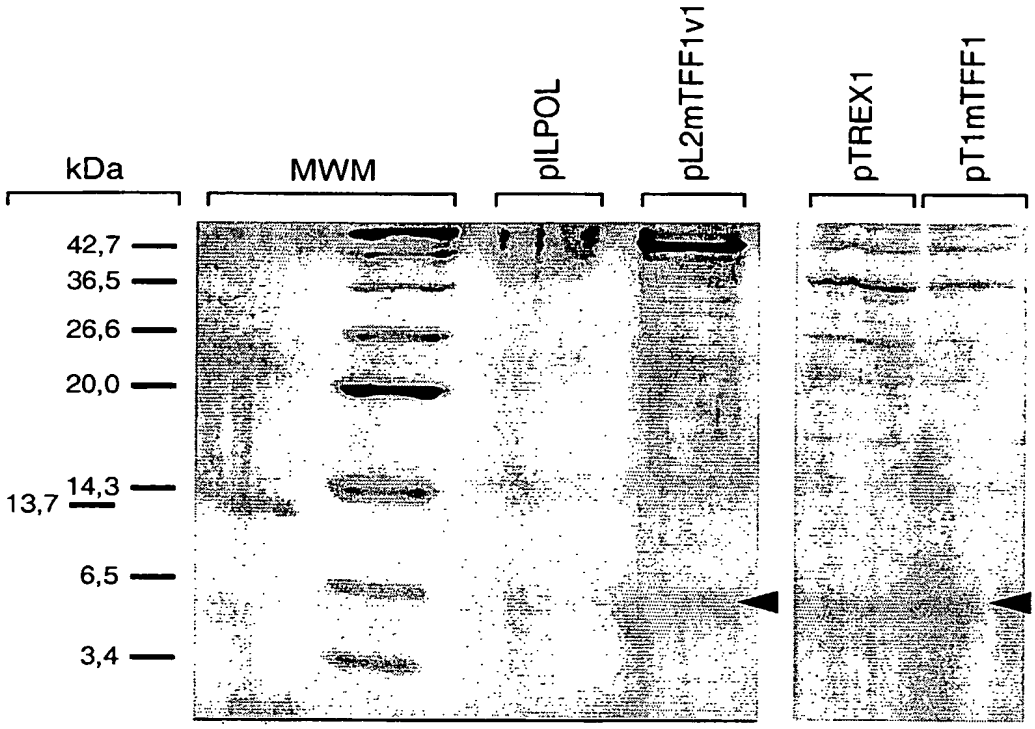

kDa
MWM
pILPOL
pL2mTFF1v1
pTREX1
pT1mTFF1
42,7
36,5
26,6
20,0
14,3
13,7
6,5
3,4

Figure 2

Distal Colon

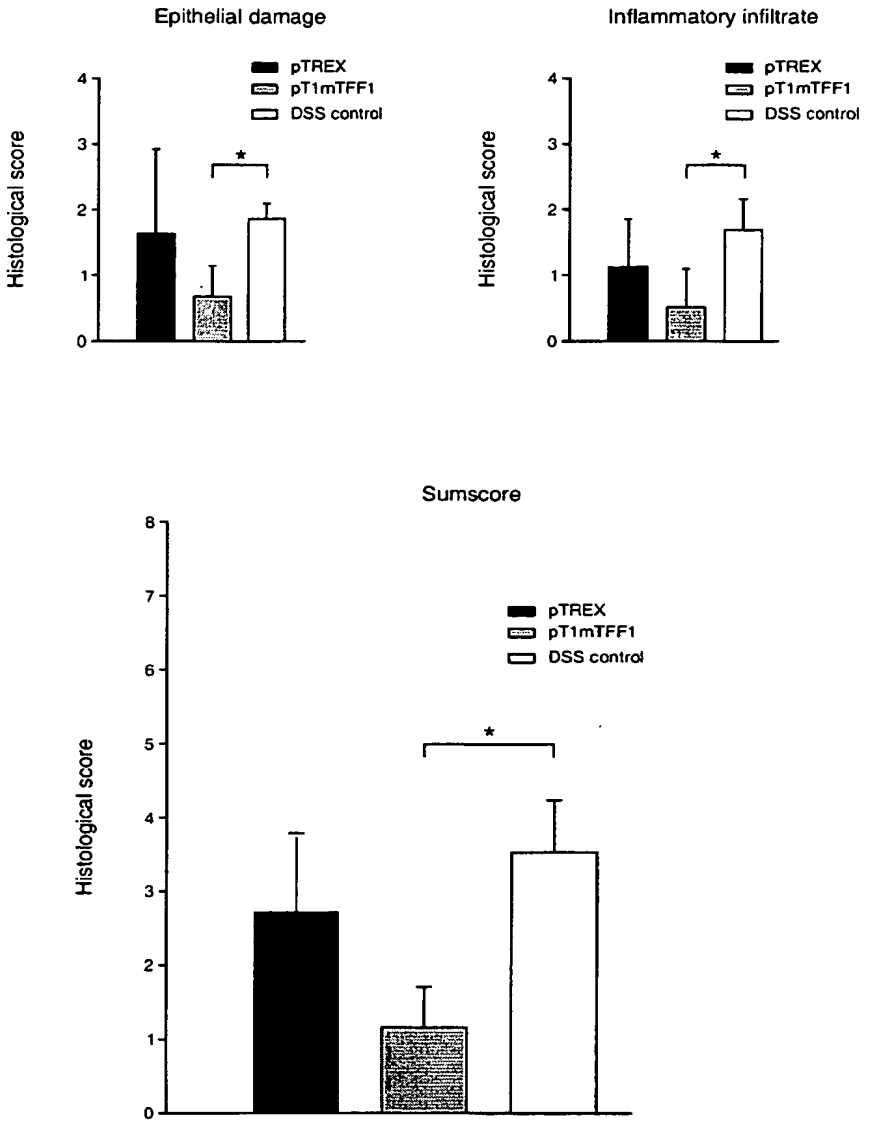


Figure 3

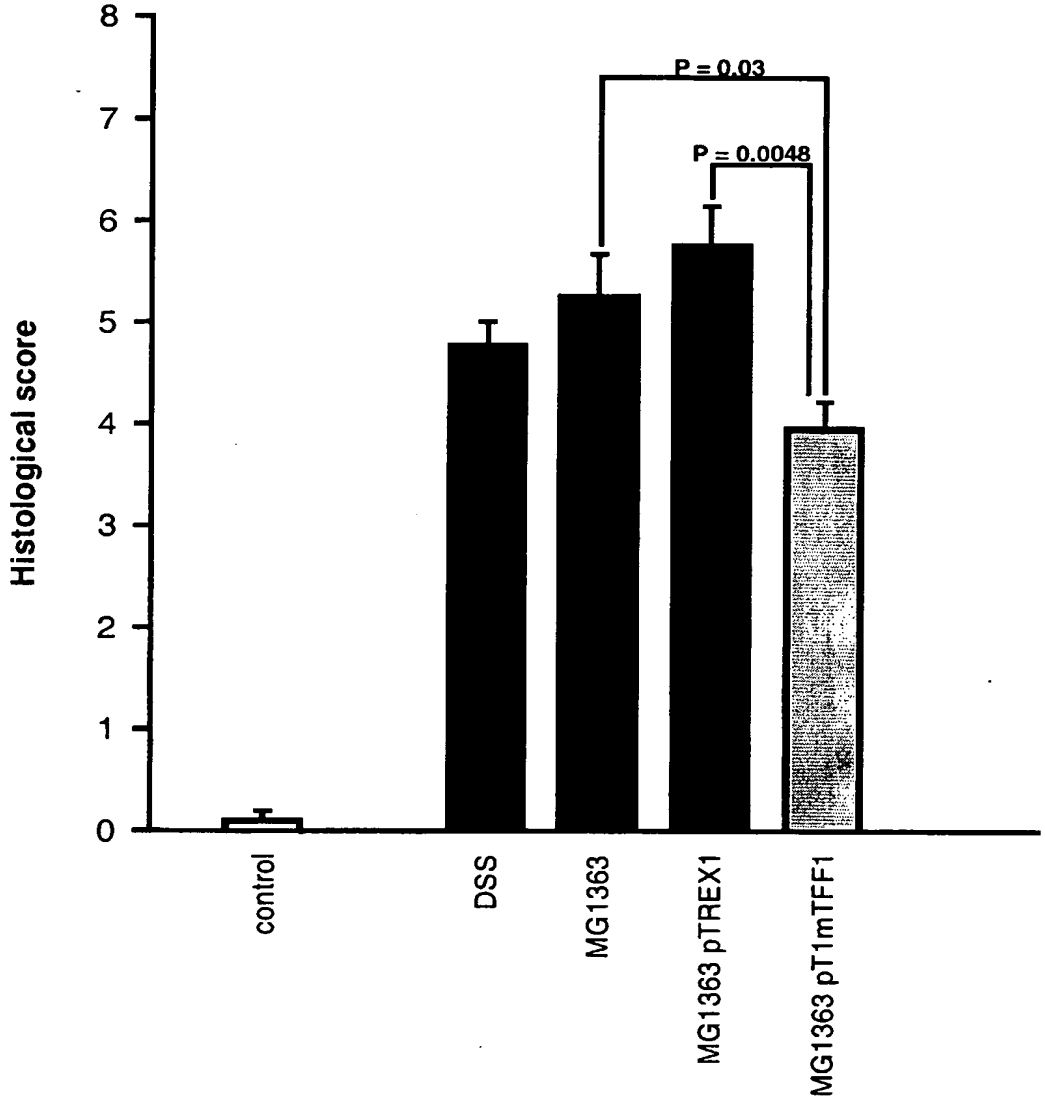
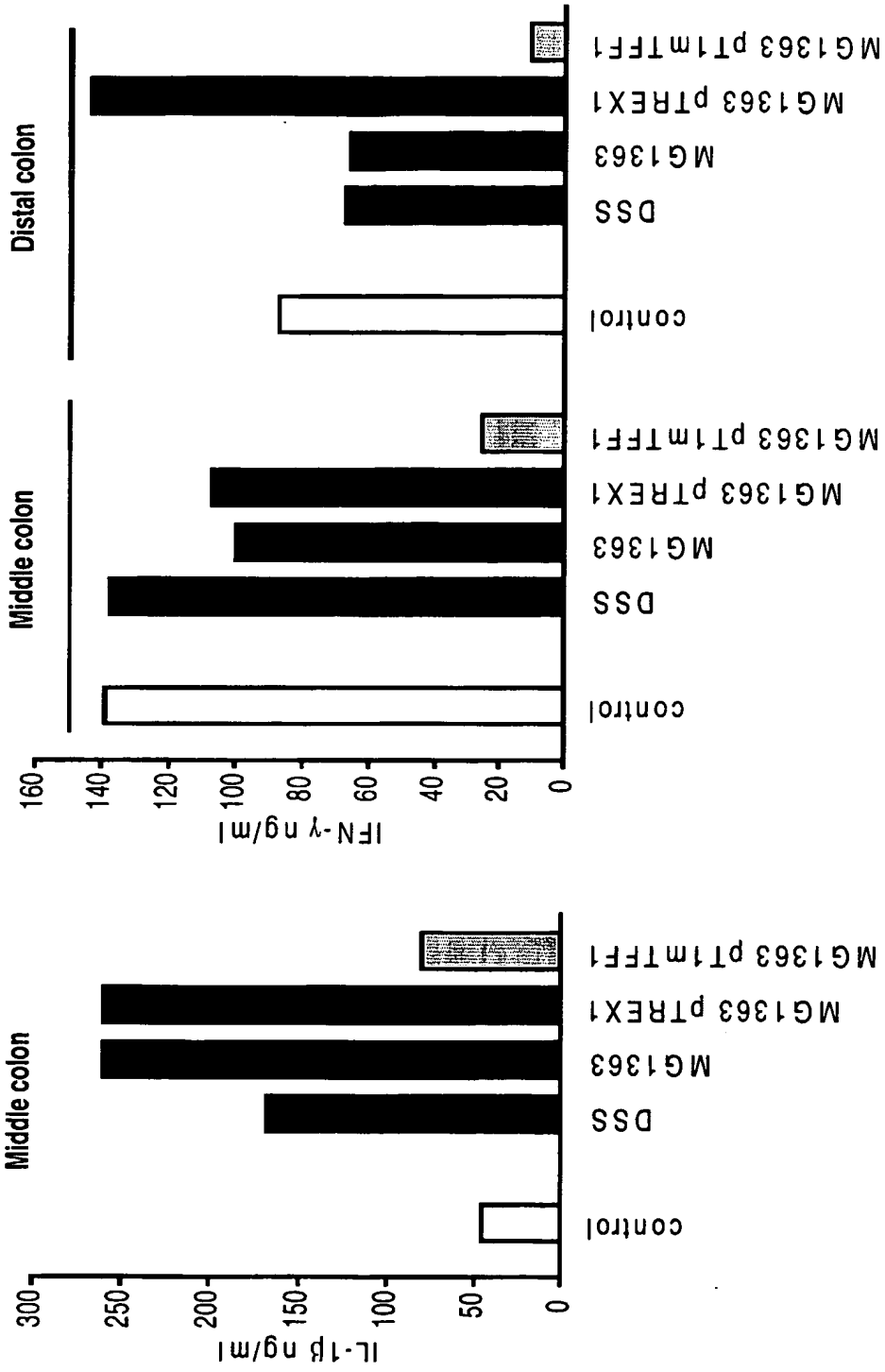

Histological score
8
7
6
5
4
3
2
1
0
P = 0.03
P = 0.0048
control
DSS
MG1363
MG1363 pTREX1
MG1363 pT1mTFF1

Figure 4


Distal colon
Middle colon
control
DSS
MG1363
MG1363 pTREX1
MG1363 pT1mTFF1
IFN-γ ng/ml
0
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40
60
80
100
120
140
160
IL-1β ng/ml
0
50
100
150
200
250
300

Figure 5

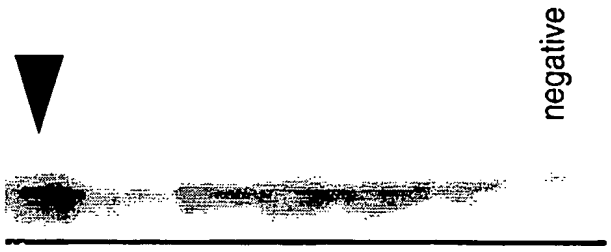

Figure 6

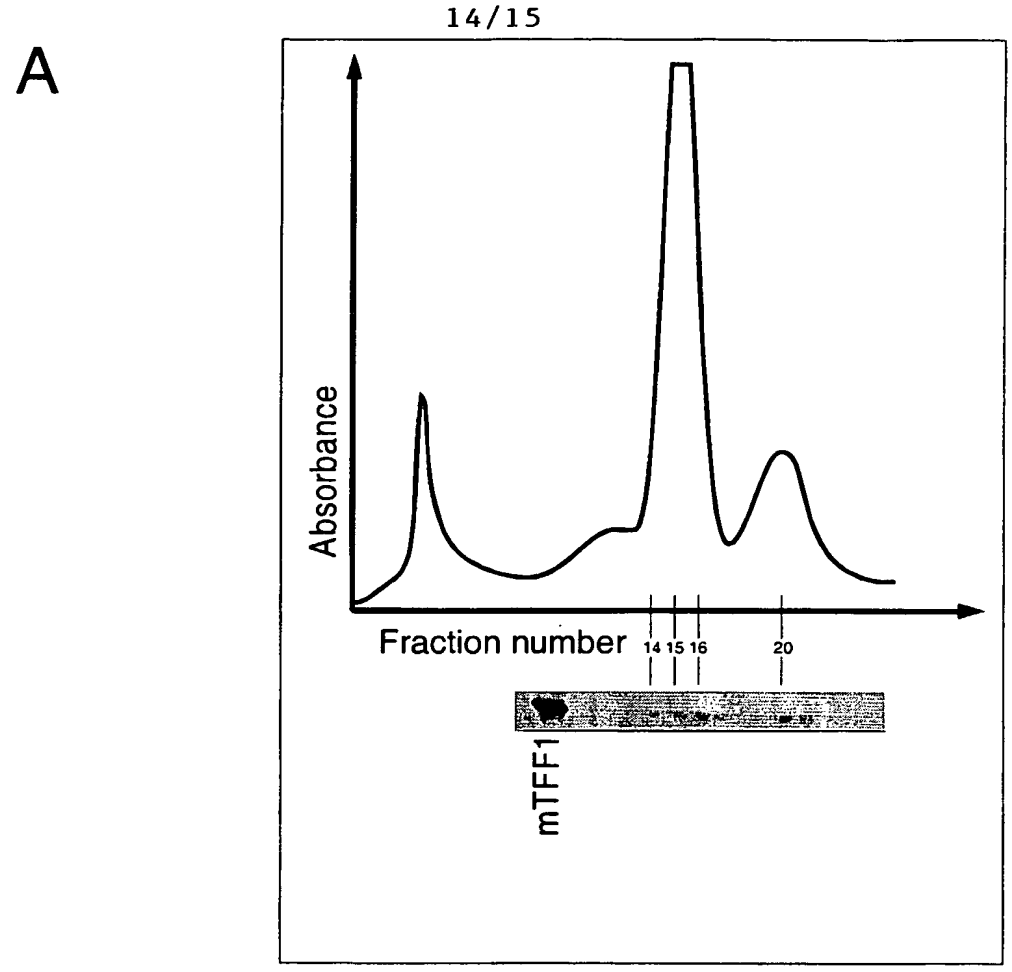
A
Absorbance
Fraction number
14 15 16
20
mTFF1
B
kDa
14,3
13,7
6,5
3,4
reducing
non reducing

Figure 7

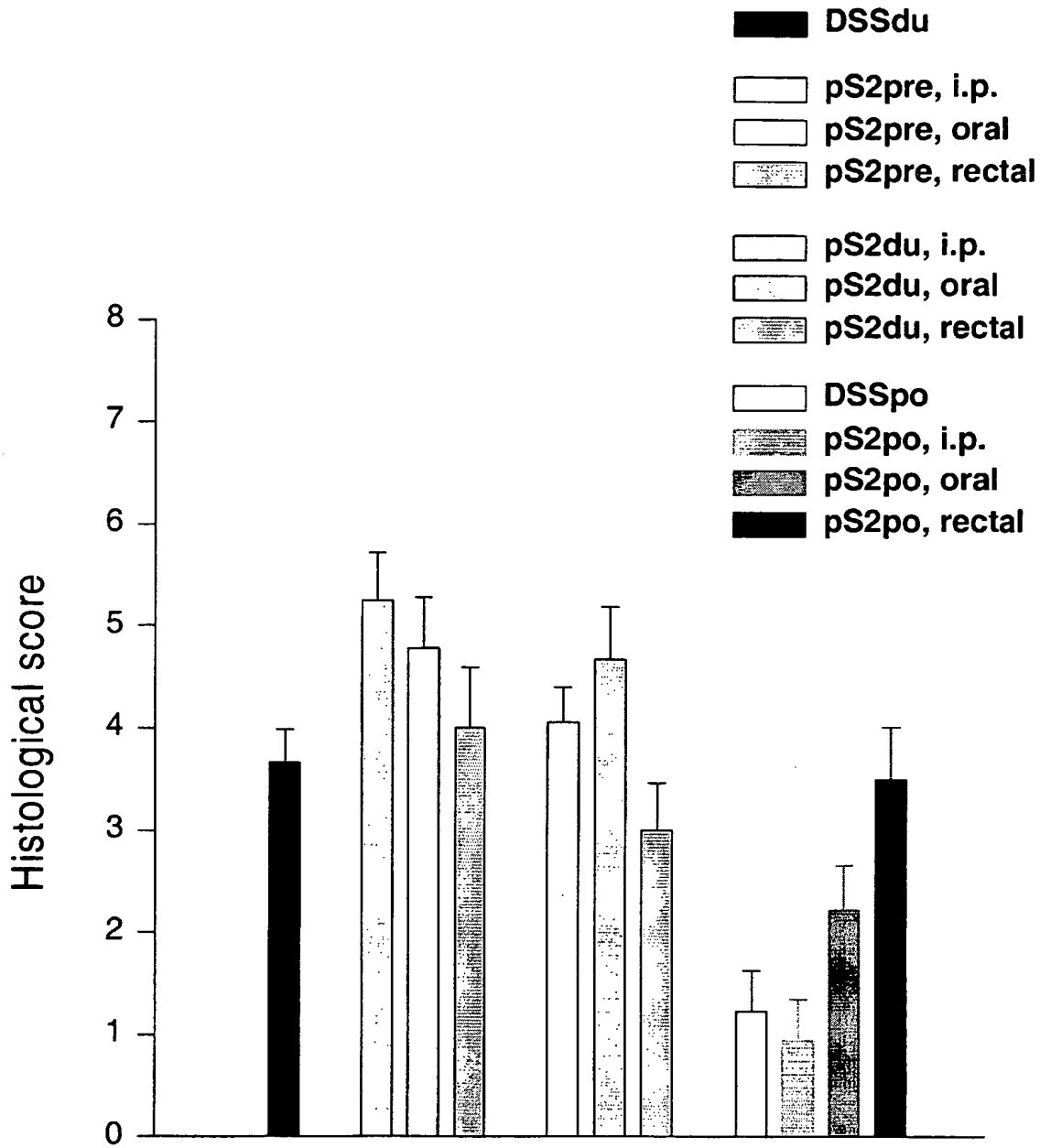
DSSdu
pS2pre, i.p.
pS2pre, oral
pS2pre, rectal
pS2du, i.p.
pS2du, oral
pS2du, rectal
DSSpo
pS2po, i.p.
pS2po, oral
pS2po, rectal
Histological score
0
1
2
3
4
5
6
7
8

Figure 8

INTERNATIONAL SEARCH REPORT

Interna[redacted]al Application No
PCT[redacted]00/06343

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/16 C12N15/74 C12N1/21 C07K14/575 A61K38/22
//(C12N1/21,C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12R A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 38712 A (GEN HOSPITAL CORP ;PODOLSKY DANIEL K (US)) 23 October 1997 (1997-10-23) cited in the application page 10, line 9 -page 11, line 25 page 21, line 24 -page 22, line 2	1,17
X	WO 92 14837 A (GEN HOSPITAL CORP) 3 September 1992 (1992-09-03) cited in the application page 15, line 29 -page 16, line 5 page 17, line 17 - line 24; claims 1-23	1,17

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 2000	20/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	X.-D. TAN ET AL.: "Characterization of a putative receptor for intestinal trefoil factor in rat small intestine: Identification by in situ binding and ligand blotting" BIOCHEM. AND BIOPHYS. RES. COMMUNICATIONS, vol. 237, 1997, pages 673-677, XP002125424 ACADEMIC PRESS, NEW YORK, US cited in the application the whole document	1,17
A	W.M. WONG ET AL.: "Trefoil peptides" GUT, vol. 44, no. 6, June 1999 (1999-06), pages 890-895, XP000857616 INT. J. OF GASTROENTEROLOGY AND HEPATOLOGY, LONDON, UK cited in the application the whole document	1-18
A	O. LEFEBVRE: "The mouse one P-domain (pS2) and two P-domain (mSP) genes exhibit distinct patterns of expression" J. CELL BIOL., vol. 122, no. 1, July 1993 (1993-07), pages 191-198, XP000857706 ROCKEFELLER UNIVERSITY PRESS,NY,US; cited in the application the whole document	1-18
A	WO 97 14806 A (UNIV CAMBRIDGE TECH ;STEIDLER LOTHAR (BE); REMAUT ERIK (BE); WELLS) 24 April 1997 (1997-04-24) cited in the application the whole document	1-18
A	WO 97 09437 A (STEIDLER LOTHAR ;REMAUT ERIK (BE); WELLS JEREMY MARK (GB)) 13 March 1997 (1997-03-13) cited in the application the whole document	1-18
P,A	WO 00 23471 A (VLAAMS INTERUNIV INST BIOTECH ;FIERS WALTER (BE); STEIDLER LOTHAR) 27 April 2000 (2000-04-27) the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/[redacted]00/06343

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9738712	A	23-10-1997	AU	2726897 A	07-11-1997
			BR	9710654 A	17-08-1999
			CA	2251631 A	23-10-1997
			EP	0954330 A	10-11-1999
			JP	2000508900 T	18-07-2000
WO 9214837	A	03-09-1992	AU	1415892 A	15-09-1992
			CA	2104104 A	03-09-1992
			EP	0573544 A	15-12-1993
			MX	9200639 A	01-10-1992
			US	6063755 A	16-05-2000
WO 9714806	A	24-04-1997	AU	7315496 A	07-05-1997
			BR	9610929 A	21-12-1999
			CN	1202934 A	23-12-1998
			EP	0871748 A	21-10-1998
			JP	2000508162 T	04-07-2000
			NO	981746 A	22-06-1998
WO 9709437	A	13-03-1997	AU	6884496 A	27-03-1997
			BR	9610133 A	21-12-1999
			CN	1201493 A	09-12-1998
			EP	0848756 A	24-06-1998
			JP	11511983 T	19-10-1999
			NO	980976 A	06-05-1998
			NZ	316580 A	29-09-1999
WO 0023471	A	27-04-2000	AU	6340199 A	08-05-2000

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
VIB-013-PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/06343	05/07/2000	05/07/1999
International Patent Classification (IPC) or national classification and IPC C12N15/16		
Applicant VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIO.et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
25/01/2001	21.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Sommerfeld, T Telephone No. +49 89 2399 7197

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/06343

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-23 as originally filed

Claims, No.:

1-18 as originally filed

Drawings, sheets:

1/15-15/15 as originally filed

Sequence listing part of the description, pages:

1-9, filed with the letter of 20.10.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 10, 11.

because:

   ☒ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-9, 12-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-9, 12-18
Industrial applicability (IA)	Yes:	Claims	1-9, 12-18
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The examination is being carried out on the following application documents:

Text for the Contracting States:
AT BE CH DE DK ES FI FR GB GR IT IE LI LU MC NL PT SE

Description, pages:
1-23 as originally filed

Claims, No.:
1-18 as originally filed

Drawings, sheets:
1/15-15/15 as originally filed

In this report reference is made to the following documents:

D1: WO 97 38712 A (GEN HOSPITAL CORP ;PODOLSKY DANIEL K (US)) 23 October 1997 (1997-10-23) cited in the application
D2: WO 92 14837 A (GEN HOSPITAL CORP) 3 September 1992 (1992-09-03) cited in the application
D3: X.-D. TAN ET AL.: 'Characterization of a putative receptor for intestinal trefoil factor in rat small intestine: Identification by in situ binding and ligand blotting' BIOCHEM. AND BIOPHYS. RES. COMMUNICATIONS, vol. 237, 1997, pages 673-677, XP002125424 ACADEMIC PRESS, NEW YORK, US cited in the application
D4: W.M. WONG ET AL.: 'Trefoil peptides' GUT, vol. 44, no. 6, June 1999 (1999-06), pages 890-895, XP000857616 INT. J. OF GASTROENTEROLOGY AND HEPATOLOGY, LONDON, UK cited in the application
D6: WO 97 14806 A (UNIV CAMBRIDGE TECH ;STEIDLER LOTHAR (BE); REMAUT ERIK (BE); WELLS) 24 April 1997 (1997-04-24) cited in the application

1. Introduction

The present application discloses methods to deliver recombinant trefoil peptides to the human or animal gastro-intestinal tract (eg. for therapy of inflammatory bowel disease) through administration of gram-positive bacteria (Lactococcus) which express the desired peptide.

2. Section III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 10 and 11 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

3. Section V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3.1. See also section VIII.

3.2. Recombinant expression of trefoil peptides in gram-positive bacteria and the administration of such bacteria to a living organism had not been disclosed in the available prior art.
Thus the subject-matter of claims 1-9 and 12-18 is novel (Art. 33(2) PCT).

3.3. However, the use of non-invasive bacteria (specifically gram-positive bacteria such as Lactococcus (eg. L. lactis) to deliver polypeptides in the body had already been disclosed in D6. Furthermore, it was already known from several prior art documents (eg. D1, D2, D3, D4) that the administration of trefoil peptides was useful for the treatment of inflammatory gastrointestinal disorders. The person skilled in the art, motivated to develop alternative and advantageous ways to deliver said peptides to the gastro-intestinal tract, would thus merely combine the teaching of documents D6 and any of documents D1 to D4 to arrive at the invention without the need for an inventive skill.
Thus the subject-matter of claims 1-9 and 12-18 lacks an inventive step (Art. 33(3) PCT).

4. Section VII

Certain defects in the international application

4.1. Claim 1 is directed to a recombinant microorganism delivering a trefoil peptide in vivo.

A recombinant microorganism could be any bacteria or virus, transformed with any recombinant DNA. However the present application just discloses delivery of trefoil peptides by gram-positive bacteria and most specifically Lactococcus transformed with DNA encoding for said peptides. It is a priori not likely that any microorganism will be suitable for this purpose.

Thus this claim is in its whole scope not sufficiently disclosed (Art. 5 PCT).

5. Section VIII

Certain observations on the international application

5.1. Claim 1 is directed to a recombinant microorganism delivering a trefoil peptide in vivo.

The wording "delivering" is unclear in the context of this claim: it could be referring to protein production or secretion or transport, each of these processes involving different mechanisms. It is also unclear from this claim where the trefoil peptide should be delivered to (into an animal, an organ, a culture medium, a cell, or else?) and what in vivo refers to (to the recombinant microorganism or to the acceptor organism?).

Claim 1 thus seriously lacks clarity (Art. 6 PCT).

In order that a meaningful opinion can be given the claim has been interpreted as: "A recombinant microorganism capable of delivering a trefoil peptide into the gastro-intestinal tract of a human or animal".

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
VIB-013-PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 06343	05/07/2000	05/07/1999
Applicant		
VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIO....		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1a

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT 00/06343

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/16 C12N15/74 C12N1/21 C07K14/575 A61K38/22
//(C12N1/21,C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12R A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 38712 A (GEN HOSPITAL CORP ;PODOLSKY DANIEL K (US)) 23 October 1997 (1997-10-23) cited in the application page 10, line 9 -page 11, line 25 page 21, line 24 -page 22, line 2	1,17
X	WO 92 14837 A (GEN HOSPITAL CORP) 3 September 1992 (1992-09-03) cited in the application page 15, line 29 -page 16, line 5 page 17, line 17 - line 24; claims 1-23	1,17

-/--

[X] Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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